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REVIEW ARTICLE

Current Concepts and Controversies in Prion Immunopathology

Mathias Heikenwalder, Marco Prinz,¹ Frank L. Heppner, and Adriano Aguzzi*

Institute of Neuropathology, University Hospital of Zürich CH-8091 Zürich, Switzerland

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Abstract

Scrapie in sheep and new variant Creutzfeldt-Jakob disease in humans are typically initiated by extracerebral exposure to prions. Both exhibit early prion accumulation in sites of the peripheral lymphoreticular system, such as splenic or lymph nodal germinal centers. In germinal centers, follicular dendritic cells (FDCs), whose development and maintenance depend on lymphotoxin and tumor necrosis factor signaling, are believed to be the main cell type for efficient prion replication in the periphery. Here, we discuss the molecular requirements for prion replication competence in stromal and lymphoid compartments of lymphoid organs. In addition, we examine the preconditions of transepithelial passage of prions in the mucosal-associated lymphoid system. Our results suggest that under specific conditions, efficient prion replication in mesenteric and inguinal lymph nodes is possible in the absence of mature FDCs. M cells are a plausible candidate for the mucosal portal of prion infection.

Index Entries: Immune system; prions; follicular dendritic cells (FDCs); lymphotoxin (LT); tumor necrosis factor (TNF).

Introduction

Prion diseases are lethal, transmissible, neurodegenerative conditions. The causative agent was proposed to be identical with prion protein (PrP)^{Sc}, a pathological conformer of the cellular protein PrP^C encoded by the cellular gene *Prnp* (Prusiner, 1982). PrP^C is expressed in many sites, notably including secondary lymphoid organs. Peripheral inoculation routes are likely to initiate most forms of spongiform encephalopathies such as sheep scrapie, bovine spongiform encephalopathy (BSE), iatrogenic Creutzfeldt-Jakob disease (iCJD) and variant CJD (vCJD). Also, intracerebral (ic) or peripheral administration of prions to mice induces a rise of infectivity in spleen and in other lymphoid organs long before the development of neurological symptoms and neuropathological changes. Intraperitoneal (ip)

inoculation has been used extensively to study the pathogenesis of transmissible spongiform encephalopathies because it causes rapid accumulation of infectivity in secondary lymphoid organs (Clarke and Haig, 1970; Hill et al., 1997; Hilton et al., 1998). The question of which compartments within lymphoreticular tissues support prion replication is of relevance to public health: Contamination with vCJD prions of germinal centers in lymph nodal and tonsillar follicles, for example, might call for precautionary measures in the handling and sterilization of surgical instruments. Also in sporadic CJD (sCJD), prions appear to be much more prevalent in extracerebral tissues than previously appreciated (Glatzel et al., 2003).

Tumor necrosis factor (TNF) and homotrimeric lymphotoxin α (LT α) signal through TNFR1, whereas membrane-bound LT α/β heterotrimers signal

*Author to whom all correspondence and reprint requests should be addressed. E-mail: adriano@pathol.unizh.ch

¹Present address: Institute of Neuropathology, Georg-August-University Göttingen, Göttingen, Germany.

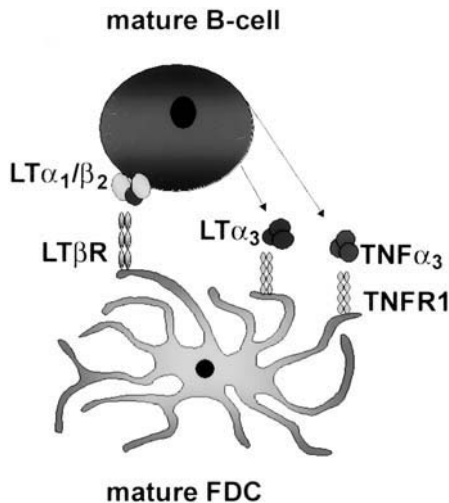


Fig. 1. Schematic drawing of signals provided by mature B cells essential for maturation and maintenance of FDCs. Membrane-bound $LT\alpha_1/\beta_2$ heterotrimers expressed on B cells signal through $LT\beta R$ expressed on mature FDCs. Soluble homotrimeric $LT\alpha_3$ and $TNF\alpha_3$ secreted by B cells signal through $TNFR1$ expressed on mature FDCs.

through $LT\beta R$ (Ware et al., 1995) (Fig. 1). $TNFR1$ and $LT\beta R$ signaling is necessary for development and maintenance of secondary lymphoid organs (Rennert et al., 1996; Koni et al., 1997; Korner et al., 1997; Futterer et al., 1998) and their proper microarchitecture. $LT\beta R$ signaling is also required for maturation and maintenance of follicular dendritic cells (FDCs), which are thought to be essential for prion replication and for accumulation of disease-associated PrP^{Sc} within secondary lymphoid organs. Inhibition of the $LT\beta$ signaling pathway with a soluble receptor, which depletes FDCs (Mackay and

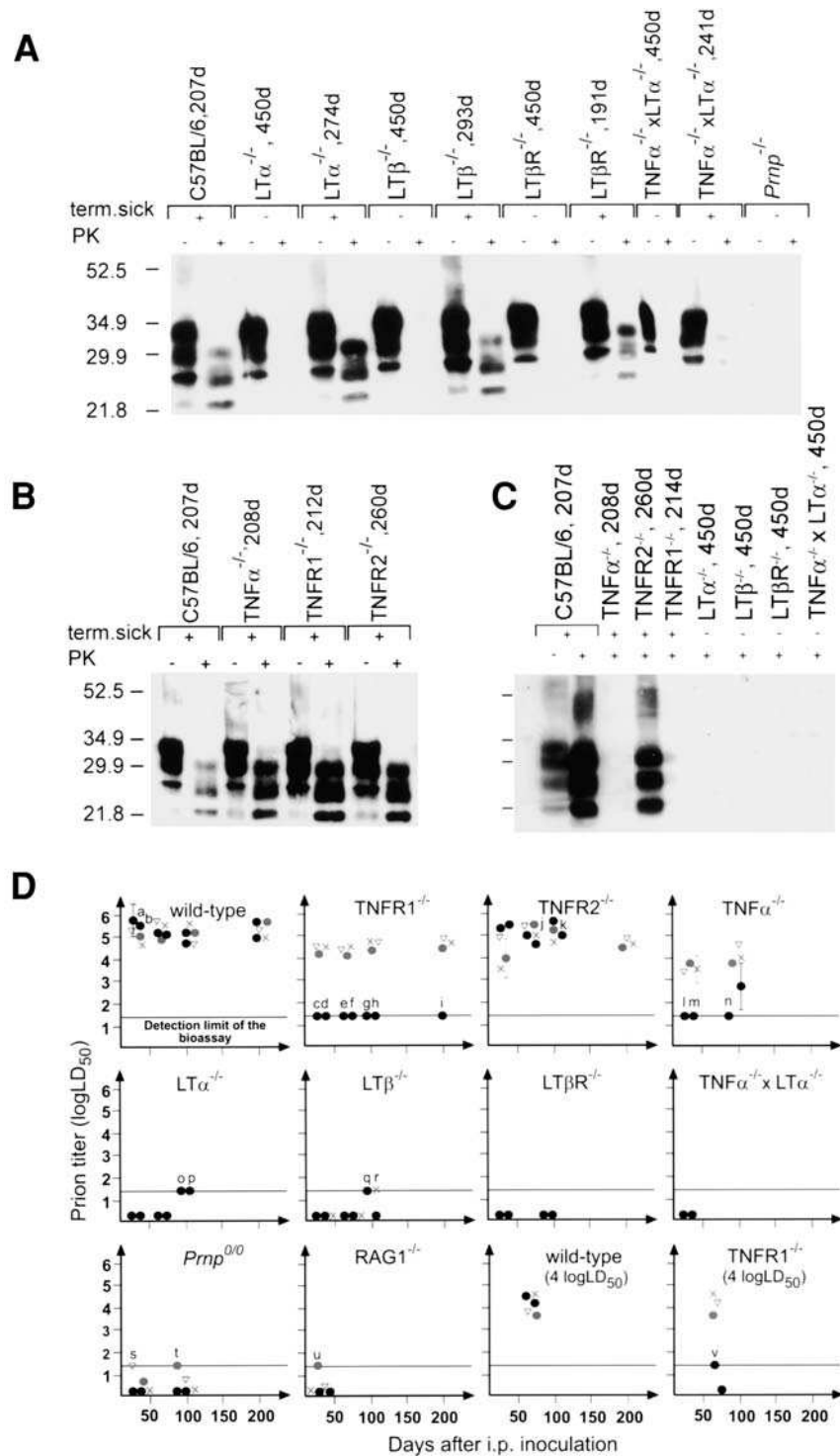
Browning, 1998), abolishes prion replication in spleens and prolongs the latency of scrapie after ip challenge (Montrasio et al., 2000). B-cell-deficient μMT mice (Kitamura et al., 1991) are resistant to prions ip (Klein et al., 1997), most likely because of impaired FDC maturation (Klein et al., 1998; Montrasio et al., 2001). Accordingly, the relative distance between FDCs and splenic nerves controls the velocity of prion neuroinvasion (Prinz et al., 2003a). In addition to FDCs, PrP^C -expressing hematopoietic cells are required for efficient lymphoreticular prion propagation (Kaeser et al., 2001).

In recent years we have studied peripheral prion pathogenesis in mice lacking $TNF\alpha$, $LT\alpha/\beta$, or their receptors. We found that ablation of $LT\beta R$ signaling prevents peripheral pathogenesis (Montrasio et al., 2000; Prinz et al., 2002), whereas ablation of $TNFR1$ signaling prevents prion pathogenesis in spleen but not in lymph nodes, despite the absence of FDCs. Moreover we have investigated some of the preconditions of transepithelial passage of prions, identifying M cells as a plausible candidate for the mucosal portal of prion infection.

Lymphoid Microarchitecture and Efficient Peripheral Prion Replication

Even though FDCs are believed to be the key cell type for efficient prion replication, mice devoid of mature FDCs did show prion titers in secondary lymphoid organs? The absence of $TNFR1$ and $TNF\alpha$ preserved susceptibility to peripheral prion challenge, whereas deletion of LT signaling components confers high resistance to peripheral prion infection, as detected by Western blot analysis and transmission experiments into *tga20* indicator mice (Fig. 2).

Fig. 2. (see opposite page) Western blot analysis of brains and spleens, as well as determination of prion infectivity titers in spleens and lymph nodes, of scrapie-challenged TNF - and LT -deficient mice. (A,B) Western blots of brain material electrophoresed natively (–), or after digestion with proteinase K (PK) (+). Large amounts of PK-resistant prion protein (PrP^{Sc}) were detected in the brain of all mice that had developed scrapie (terminal sick), independently of the genotype. Clinical healthy mice deficient in LT signaling showed no PrP^{Sc} accumulation, excluding subclinical scrapie (A). (C) Western blots of spleen homogenates electrophoresed natively (–) or after digestion with proteinase K (+). $TNFR1^{-/-}$ and $TNF\alpha^{-/-}$ mice accumulated lower amounts of PrP^{Sc} as compared to $TNFR2^{-/-}$ and wild-type mice. No PrP^{Sc} was detected in the spleens of clinical healthy LT -deficient mice. (D) Prion infectivity in lymphoid organs. Titers were determined in spleens (dark circles, ●), inguinal (light circles, ○), mesenteric (crosses, X), and cervical (triangles) lymph nodes at the time points indicated below the graphs. Mice were inoculated ip with 6 $\log LD_{50}$ or 4 $\log LD_{50}$ of scrapie prions as indicated. Standard deviations within groups are drawn only when exceeding $\pm 0.75 \log LD_{50}$. (a,b) In each of two separate transmissions, 1 out of 4 *tga20* mice died 24 h after inoculation, probably because of intracerebral bleeding after injection. (j,k) Intercurrent death during incubation time. Symbols on the abscissa indicate prion titers below detection limit (none of the four indicator mice developed scrapie). If one or more indicator mice survived <180 dpi, or the mean incubation time was >120 d, titer was assumed to be close to the detection threshold of the bioassay. For these Fig. 2. (con-



tinued) samples (labeled with lowercase letters) the numbers of animals succumbing to scrapie out of four inoculated *tga20* mice and incubation time (in days) to terminal scrapie were as follows: (c) 1/4 (111), (d) 3/4 (93, 117, 132), (e) 2/4 (90, 95), (f) 1/4 (126), (g) 3/4 (112, 123, 128), (h) 4/4 (106, 115, 124, 136), (i) 3/4 (89, 102, 119), (l) 4/4 (115, 115, 126, 135), (m) 1/4 (98), (n) 2/4 (74, 78), (o) 2/4 (125, 151), (p) 2/4 (131, 193), (q) 3/4 (119, 125, 152), (r) 2/4 (109, 117), (s) 1/4 (108), (t) 1/4 (71) (u) 3/4 (80, 96, 98). (v) 2/4 (98, 98).

These differential effects on scrapie susceptibility were surprising, as all of these defects (except TNFR2^{-/-}) abolish FDCs, which were thought to be crucial for pathogenesis. However, LT/TNF family members play distinct roles in lymph node induction and development (Fu and Chaplin, 1999): Lymphoid organ architecture, including organization of T and B cell zones, is more severely impaired in LT- than in TNF-deficient mice (Fu and Chaplin, 1999). Because LT and TNF signaling defects might potentially affect several immune cell lineages, we have performed additional adoptive fetal liver cell transfer studies that complement the gene ablation experiments.

In *Prnp*^{0/0} mice grafted with TNFR1^{-/-} fetal liver cells (FLCs), high infectivity loads (3.4–4.2 logLD₅₀) were detectable in lymph nodes but not spleens, strongly indicating a role of TNFR1-deficient hematopoietic cells in efficient prion propagation within lymph nodes. Conversely, lymph nodes of TNFR1^{-/-} mice grafted with *Prnp*^{0/0} FLCs contained substantial amounts of infectivity. Therefore, stromal cells also contribute to the capability of TNFR1^{-/-}-deficient lymph nodes to replicate prions.

Reciprocal reconstitution of wild-type and *Prnp*^{0/0} mice showed once again that efficient lymphoreticular prion propagation required PrP^C expression in both stromal and hematopoietic compartments (Blättler et al., 1997; Kaeser et al., 2001).

These results imply that prion replication can take place in secondary lymphoid organs even in the absence of mature FDCs and that other cell types can maintain replication of prions to titers that are similar to those of wild-type mice. Moreover, prion pathogenesis in the lymphoreticular system can be topographically compartmentalized, and lymph nodes can represent an important reservoir of prion infectivity during disease.

This hypothesis is strengthened by the fact that even in wild-type lymph nodes, bright PrP signals outside FDC networks colocalized with a subset of ER-TR9- and MOMA-1-positive cells (Prinz et al., 2002).

TNF α signaling through TNFR1 is required for proper homing of macrophages to the splenic marginal zone, and their absence can cause strong aberrations in macrophage subsets. However, MOMA-1⁺ and ER-TR9⁺ macrophages were normally distributed in the subcapsular area of TNFR1^{-/-} and TNF α ^{-/-} lymph nodes (Pasparakis et al., 2000) but were strongly disturbed in mesenteric lymph nodes of LT β ^{-/-} mice. It was suggested that FDC precursor

cells in TNFR1^{-/-} mice could be located in the germinal center marginal zone and could interact with macrophages there. In addition, this study showed that to the defects in follicular structure, TNFR1^{-/-} mice exhibit defects in the formation of the splenic but not the lymph node macrophage populations, which is in contrast to all LT knockout mice. The investigators concluded that the primary defect in the lymphoid phenotype of TNFR1^{-/-} mice is the failure of possible FDC precursors to migrate through the disorganized marginal sinus and to home properly into the lymph node follicular areas where they would promote the formation of B-cell follicles and germinal centers (Pasparakis et al., 2000).

If the distribution of macrophages is important for peripheral prion pathogenesis, these histoarchitectural differences might account for the differences in splenic versus lymph nodal prion load of infected TNFR1^{-/-} and TNF α ^{-/-} mice. Primary and secondary follicles might be functionally different in spleen vs mesenteric lymph nodes of TNFR1^{-/-} mice (Fu et al., 1997). However, we did not identify morphological differences between splenic and mesenteric germinal centers, and the TNFR1^{-/-} line used here (Rothe et al., 1993) did not show abnormal germinal center responses after infection with vesicular stomatitis virus (Karrer et al., 2000).

As described repeatedly (Klein et al., 1997; Kaeser et al., 2001; Prinz et al., 2002), B-cell-deficient mice were entirely resistant to ip-administered prions, whereas TNF/LT-deficient mice were partially resistant. These findings differ from those reported by Mabbott and colleagues (2000) and suggest that the importance of B lymphocytes in prion pathogenesis might go beyond their role in FDC maintenance.

The unexpected finding of high prion titers in inguinal, mesenteric, and cervical lymph nodes of TNF-deficient—but not in mesenteric lymph nodes of LT β -deficient—mice indicates that prion replication within secondary lymphoid organs is LT β R dependent yet may occur in the absence of mature FDCs and functional germinal centers.

Therefore, cell types other than mature FDCs participate in the process of prion replication/accumulation in lymph nodes and, probably, in spleens. Because marginal zone macrophages might entertain close contacts to immature FDCs in the marginal zone, whose presence was postulated for the TNFR1^{-/-} mice (Pasparakis et al., 2000), and also interact with marginal zone B cells, this cell type is certainly a candidate for supportive effects in the process of prion uptake and replication.

However, this interpretation has one caveat: Immunofluorescence detects PrP rather than infectivity and does not differentiate unequivocally between PrP^C and PrP^{Sc}. Therefore, further studies will need to focus on whether macrophage ablation, that is, using macrophage-specific suicide transgenes, can suppress the infectibility of TNF-deficient lymph nodes.

Moreover, these findings are at striking variance with reports that LT $\beta^{-/-}$ mice are fully susceptible to infection with CJD prions (Rothe et al., 1993) and that TNF $\alpha^{-/-}$ mice peripherally challenged with ME7 prions are largely protected (Mabbott et al., 2000). These and other discrepancies have been attributed to the use of different prion strains in these studies. This may well be the case, but our results indicate that resistance in each mouse strain is dose dependent and can always be overridden. Therefore, challenge with one single size of inoculum, as done in other studies, might yield misleading results.

Invasion of lymphoid organs by prions occurs very rapidly after peripheral inoculation, and consistently high infectivity titers are detected until terminal disease. Lymphoinvasion most likely plays an important role in the pathogenesis of vCJD, as prion infectivity can be detected in the tonsils of virtually every vCJD patient (Bruce et al., 2001; Wadsworth et al., 2001). After lymphoinvasion, neuroinvasion occurs via autonomic nerves (Cole and Kimberlin, 1985; Race et al., 2000; Glatzel et al., 2001), but the nexus between germinal centers and nerves is still elusive. By virtue of their mobility, macrophages may represent a plausible candidate for transport of prion infectivity from germinal centers to sympathetic nerve terminals.

How could a possible prion amplification in macrophages be reconciled with their apparent protective role, at least in the very early phase of prion pathogenesis (Beringue et al., 2000)? Maybe the action of macrophages is dose dependent: Small inocula might be destroyed by phagocytosis, whereas larger inocula cannot be digested and will be transported or amplified. Alternatively, the absence of TNFR1 might interfere directly with the interaction of macrophages and prions, as ablation of TNF signaling reduces the phagocytic ability of macrophages in several infectious models (Yap et al., 1998). Nevertheless, the fact that a cell type other than mature FDCs is involved in prion replication and accumulation within secondary lymphoid organs might help to develop postexposure prophylaxis strategies aimed at blocking prion neuroinvasion.

The Pathway of Orally Administered Prions

Upon oral challenge, an early rise in prion infectivity can be observed in the distal ileum of infected organisms: This applies to several species but was investigated most extensively in sheep (Wells et al., 1994; van Keulen et al., 1999). There, Peyer's patches (PPs) acquire strong immunopositivity for the PrP. Immunohistochemical stains with antibodies to the PrP typically reveal a robust signal in primary B-cell follicles and germinal centers, which roughly colocalizes with the complement receptor CD35 in a wide variety of secondary lymphoid organs, including appendix and tonsils (Hill et al., 1997). Although conventional light microscopy does not allow differentiation between PrP^C and PrP^{Sc}, Western blot analysis has not left any doubt about the fact that PPs do accumulate the disease-associated form of the PrP.

The latter is true also in the mouse model of scrapie, which is being used as a convenient experimental paradigm by many laboratories, including ours. Administration of mouse-adapted scrapie prions (Rocky Mountain Laboratory or RML strain, originally derived from the Chandler sheep scrapie isolate) induces a surge in intestinal prion infectivity as early as a few days after inoculation (Prinz et al., 2003b).

All of the above evidence conjures the suggestion that PPs might represent a portal of entry for orally administered prions on their journey from the luminal aspect of the gastrointestinal tube to the central nervous system. However, the question as to whether the same applies to BSE-affected cattle has been answered less definitely.

In a monumental study of BSE pathogenesis in cattle, carried out at the UK Veterinary Laboratory Agency, cows of various ages were fed with 100 g, 10 g, 1 g, or 100 mg of brain homogenate derived from BSE-sick cows (Bradley, 2000). A large variety of tissues were taken at various points in time, homogenized, and transmitted to indicator to assess their prion content. This study was designed to be performed over a time frame of more than a decade and was still under way at the time of this writing: It has uncovered a transient surge in infectivity in the distal ileum of cows at approx 6 mo postinfection. Infectivity then subsides, but it appears to return to the terminal ileum at the end stages of disease, maybe by means of some sort of retrograde transport (Wells et al., 1998). Although this was not

formally confirmed, it appears likely that PPs are the sites of prion accumulation in the gastrointestinal tract of cattle challenged orally with prions.

Transepithelial Enteric Passage of Prions: A Role for M Cells?

We have set out to investigate some of the preconditions of transepithelial passage of prions. Membranous epithelial cells (M cells) are key sites of antigen sampling for the mucosal-associated lymphoid system (MALT) and have been recognized as major ports of entry for enteric pathogens in the gut via transepithelial transport (Neutra et al., 1996). Interestingly, maturation of M cells is dependent on signals transmitted by intraepithelial B cells. The group of Jean-Pierre Kraehenbuhl (Lausanne) has developed efficient *in vitro* systems, in which epithelial cells can be instructed to undergo differentiation to cells that resemble M cells by morphological and functional-physiological criteria (Kerneis et al., 1997). Therefore, we investigated whether M cells are a plausible site of prion entry in a coculture model (Kerneis et al., 1997) (Fig. 3). Colon carcinoma cells (line Caco-2) were seeded on the upper face of transwell filters and cultured until confluency was reached. Next, B-lymphoblastoid Raji cells were added onto the lower side of the filters. Lymphoid cells migrated through the pores of the filter and settled within the epithelial monolayer, inducing differentiation of some Caco-2 cells into M cells. Successful conversion was monitored by measuring transport of fluorescein-conjugated latex beads in cocultures that exhibited a high transepithelial resistance and were therefore tight. Active transepithelial transport of beads, but not passive leakage, was blocked at 4°C. Scrapie prions were administered to the apical compartment of cocultures that combined integrity and active transport of beads. After 24 h, infectivity was determined within the basolateral compartment by bioassay with *tga20* mice, which overexpress a *Prnp* transgene and develop scrapie rapidly after infection (Fischer et al., 1996). Upon challenge with 5 logLD₅₀ of scrapie prions, we consistently recovered prions in the basolateral compartment of cocultures containing M cells, suggesting transepithelial prion transport. Even at low prion doses (3 logLD₅₀), we found infectivity in at least one M-cell-containing coculture. In contrast, there was hardly any prion transport in Caco-2 cultures without M cells (Heppner et al., 2001) (Fig. 3).

These findings indicate that M-cell differentiation is necessary and sufficient for active transepithelial prion transport *in vitro*. M-cell-dependent uptake of foreign antigens or particles is known to be followed by rapid transcytosis directly to the intraepithelial pocket, where key players of the immune system, for example, macrophages, dendritic cells, and lymphocytes (Neutra et al., 1996), are located. It has been shown that immune cells are crucially involved in the process of neuroinvasion and that (as suspected) mature FDCs, located in the PP, are critical for the transmission of scrapie from the gastrointestinal tract (Aguzzi and Heppner, 2000; Mabbott et al., 2003). Therefore, prions might exploit M-cell-dependent transcytosis to gain access to the immune system.

Although these findings suggest that M cells are a plausible candidate for the mucosal portal of prion infection, it still remains to be established whether the pathway delineated above does indeed represent the first portal of entry of orally administered prions into the body. This will necessitate *in vivo* experimentation, that is, by ablation of M cells through suicide transgenic strategies, or by M-cell-specific expression of *Prnp* transgenes. Results of such experiments might help to define specific therapeutic strategies to prevent prions from entering the immune system after oral uptake.

To this effect, it is interesting to note that a dimeric fusion protein composed of two immunoglobulin Fc_γ domains fused to two PrP^C molecules (PrP-Fc₂ fusion protein) prolongs the latency period of prion infection upon expression in transgenic mice by competing with PrP^C for PrP^{Sc} (Meier et al., 2003). This highly stable, soluble, dimeric fusion protein might serve as an effective antiprion therapeutic acting at the site of uptake, in the periphery, or even in the brains of infected individuals.

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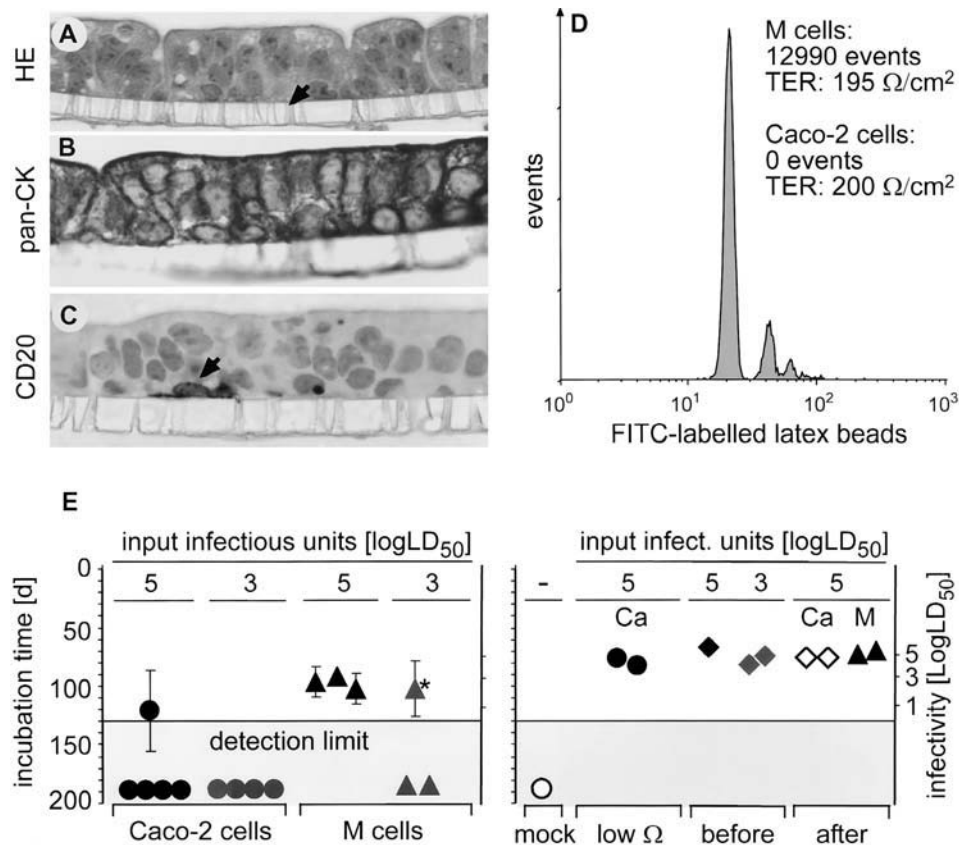


Fig. 3. Transepithelial transport of prions via M cells. Filter membranes with 3- μm pores were overlaid with epithelial cells (Caco-2). Human B-lymphoblastoid Raji cells were then cultured on the back side of the membrane. (A) Morphology of cocultures on filters, visualized by hematoxylin/eosin (HE) stains. (B) Immunohistochemical stain for cytokeratins (pan-CK) visualizing human Caco-2 epithelial cells, and (C) for the B-cell marker CD20, displaying B cells that have successfully migrated through the pores of the filter (arrow). (D) Flow cytometric analysis demonstrating active (i.e., temperature-dependent) transport of FITC-conjugated latex beads from the apical to the basolateral chamber compartment upon integration of B cells within pouches of the epithelial Caco-2 cell layer, and differentiation of M cells. Intactness of cocultures was assessed by measuring transepithelial resistance at 37°C, which was typically $>200 \Omega/\text{cm}^2$. The presence of additional peaks corresponds to aggregates of several latex beads. (E) Recovery of prion infectivity upon challenge with different prion inocula (5 [dark] or 3 [light] logLD₅₀ input infectious units) was only visible in cocultures containing M cells (triangles, lanes 3,4). No prion transport was observed in Caco-2 cultures (circles, lanes 1,2) without M cells, except in one case in which traces of infectivity were present. Controls: mock inoculum (lane 1), Caco-2 culture (Ca) after slight mechanical manipulation resulting in a transepithelial resistance of $<50 \Omega/\text{cm}^2$ (lane 2). Prion infectivity dilutions are as indicated before (positive control, lane 3,4) and after incubating with either Caco-2 (lane 5) or M-cell-containing (M) cultures (lane 6). Each symbol represents the mean incubation time in days (ordinate) of four *tga20* indicator mice until terminal disease.

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